PEPTIDOGLYCAN SYNTHESIS GENE DISRUPTION USING CRISPR/CAS9 ON EXTENDED SPECTRUM BETA LACTAMASE *Klebsiella pneumoniae* STRAIN UPM ESBLKP1

By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : Associate Professor Nurulfiza Mat Isa, PhD
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The resistance ability of several bacteria towards drugs and antibiotics has been alarming worldwide. These bacteria are known as multiple drug resistance (MDR) organisms. A group of bacteria-harbouring beta-lactamase has become increasingly resistant to the most used antibiotics. Extended Spectrum Beta Lactamase (ESBLs) producing *Enterobacteriaceae* is an emerging public health concern where ESBL *Klebsiella pneumoniae* is a significant problem in major hospitals. In Europe and the United States, *K. pneumoniae* caused 8% of total nosocomial infections in hospitals, ranking them second after *Escherichia coli*. They also caused up to 17% of urinary tract infections in both countries. Research is done to find the best, most convenient, and most economically affordable ways to fight them.

This research investigated the efficacy of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology as a novel strategy to genetically modify the genome and inhibit the growth of UPM ESBLKP1 *K. pneumoniae*. A CRISPR-Cas9 vector was constructed with the designed guide RNA (gRNA) targeted specifically to the *uppP* gene, a gene responsible for cell wall peptidoglycan synthesis, which is responsible for bacterial cell growth and protection. Three mutants, uppP1, uppP2, and uppP3, were constructed using high voltage transformation of pCasSA+CmR plasmid that carries both gRNA and Cas9 coding gene. The negative control and mutant strains were observed analysed using rich and limiting media for growth performance and lysozyme assay for cell wall integrity.

Interestingly, the negative control, $_{contrl}$*Klebsiella pneumoniae*, showed a standard growth curve, while the mutant strains showed a faster doubling rate. Meanwhile, the cell wall integrity of the modified *K. pneumoniae* was significantly reduced with mean value at alpha = 0.05 with 0.5130, 0.5160, 0.3700, and 0.2290 when compared to the negative control with Mutant uppP1, Mutant uppP2, and Mutant uppP3 analysed with

lysozyme assay respectively. Cell wall appearance was observed using Transfer Electron Microscopy (TEM). Images show positive disruption of mutants' strain cell walls. The cell wall of Mutant uppP1 appeared to be burst, and the Mutant uppP2 cell wall, on the other hand, appeared to have been snapped and clumped together; meanwhile, the Mutant cell wall of the Mutant uppP3 cell walls cannot be seen and identified. This indicates a successful modification of the *uppP* gene of all three mutants. The pathogenicity study shows that cells treated with Mutant uppP3 have a higher percentage of cell viability followed by both Mutant upp2 and Mutant uppP1 compared to wild type for all three-time frames (24h, 48h, and 72h). The developed CRISPR-Cas9 system has successfully modified the targeted *K. pneumoniae uppP* gene hence providing an opportunity to create an alternative treatment for *K. pneumoniae* infection.

Keywords: CRISPR-Cas9, ESBL *Klebsiella pneumoniae*, peptidoglycan synthesis gene, cell wall integrity, TEM

SDG: Goal 3: Good Health and Well-being; Goal 17: Partnerships for the Goal.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

GANGGUAN TERHADAP GEN SINTESIS PEPTIDOGLIKAN MELALUI KAEDAH MEKANISMA CRISPR/CAS9 KE ATAS ORGANISMA BETA LAKTAM YANG BERSPEKTUM LUAS (ESBL) JENIS *Klebsiella pneumoniae* UPM ESBLKP1

Oleh

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Keupayaan daya tahan beberapa bakteria terhadap ubat-ubatan dan antibiotik telah membimbangkan para penyelidik dan ahli perubatan di seluruh dunia. Bakteria ini dikenali sebagai organisma tahan pelbagai ubatan. Salah satu kumpulan bakteria ini mempunyai beta-laktam gen. Perkara ini dapat meningkat daya tahan bakteria tersebut terhadap antibiotik yang telah digunakan sebagai rawatan di hospital dan klinik. Bakteria dari keluarga *Enterobacteriaceae* yang menghasilkan enzim beta laktam turut mencetuskan kebimbangan terhadap kesihatan awam. Manakala, ESBL *Klebsiella pneumoniae* pula telah menjadi salah satu masalah utama di kebanyakan negara, terutamanya penyelidik dan pihak petugas kesihatan di hospital utama. Sebagai contoh, kajian lepas yang dijalankan di Eropah dan Amerika Syarikat mendapati bahawa, *Klebsiella pneumoniae* menduduki tempat kedua selepas *Escherichia coli* yang menyebabkan 8% daripada jumlah jangkitan nosokomial di hospital. *Klebsiella pneumoniae* juga didapati menyebabkan sehingga 17% jangkitan di saluran kencing di kedua-dua negara tersebut. Kini, banyak penyelidikan telah dan sedang dijalankan bagi mencari kaedah rawatan terbaik, mudah, dan berpatutan dari segi ekonomi untuk menentang bakteria ini.

Maka, kajian ini bertujuan untuk mengkaji keberkesanan teknologi *Clustered Regularly Interspaced Short Palindromic Repeats* (*CRISPR*) sebagai strategi baharu untuk mengubah genom secara genetik dan menghalang pertumbuhan *Klebsiella pneumoniae* UPM ESBLKP1. Vektor CRISPR-Cas9 dibina bersama dengan RNA panduan (gRNA) yang direka khusus untuk gen *uppP*. Gen ini bertanggungjawab bagi proses sintesis peptidoglikan. Hal ini dikatakan demikian kerana dinding sel tersebut akan bertanggungjawab terhadap pembesaran bakteria ini, termasuklah pertumbuhan dan perlindungan sel. Kajian ini mengemukakan tiga mutan iaitu mutan uppP1, mutan uppP2 dan mutan uppP3 dibina. Sampel asal yang dijadikan sebagai sample kawalan;

*contrl*Klebsiella pneumoniae dan mutan diperhatikan dan dianalisis. Hasil kajian ini mendapati bahawa, prestasi pertumbuhan dan integriti dinding sel *Klebsiella pneumoniae* yang diubah berkurangan dengan signifikan pada nilai min pada alpha = 0.05 dengan perkadaran nilai min sebanyak 0,5130, 0,5160, 0,3700 dan 0,2290 untuk sampel *contrl*Klebsiella pneumoniae, Mutan uppP1, Mutan uppP2 dan Mutan uppP3 ketika dianalisis dengan kaedah lysozim.

Menariknya, sampel asal *contrl*Klebsiella pneumoniae menunjukkan graf pertumbuhan normal, sementara strain mutan menunjukkan kadar penggandaan lebih cepat akibat daripada kejayaan pengubahsuaian gen. Hasil dapatan daripada pemerhatian integriti dinding sel dengan Mikroskop Pemindahan Elektron (TEM) pula menunjukkan gangguan terhadap dinding sel strain mutan. Hal ini disebabkan oleh, dinding sel Mutan uppP1 kelihatan pecah, dinding sel Mutan uppP2 di sisi lain kelihatan terputus dan bergumpal bersama, sementara dinding sel uppP3 mutan tidak dapat dilihat dan dikenal pasti. Dapatan kajian ini telah menunjukkan kejayaan terhadap pengubahsuaian gen *uppP* bagi Mutan uppP3. Dapatan kajian terhadap patogenik pula, menunjukkan perbezaan Mutan uppP3 memberikan menyebabkan kadar kematian yang rendah kepada sel dan diikuti Mutant uppP2 dan Mutant uppP1 jika dibandingkan dengan sample asal pada ketiga-tiga tempoh masa kajian. Sistem CRISPR-Cas9 yang dikembangkan ini berjaya mengubah gen *Klebsiella pneumoniae* yang disasarkan sehingga dapat memberi peluang atau alternatif antibiotik sebagai kaedah rawatan baharu bagi jangkitan *Klebsiella pneumoniae*.

Kata Kunci: CRISPR-Cas9, ESBL *Klebsiella pneumoniae*, gen sintesis peptidoglikan *uppP*, integriti dinding sel, TEM

Matlamat Pembangunan Mampan: Matlamat 3: Kesihatan dan Kesejahteraan yang Baik; Matlamat 17: Perkongsian untuk Matlamat.

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Last but not least, well done, Nasharudin, as you can complete your study. Well done!

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

MDR	Multidrug Resistant
CDC	Centre of Disease Controls and Prevention
°C	Degree celsius
%	Percentage
OD_{600n}	Optical density at wavelength 600 nanometer
µL	Microliter
µm	Micrometer
mL	Mililter
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
gRNA	Guide RNA
HGT	Horizontal Gene Transfer
bp	Base pair
kbp	Kilo base pair
Mbp	Mega base pair
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
PROTEKT	Prospective Resistant Organisms Tracking & Epidemiology for the Ketolide Telithromycin
EDTA	Ethylenediaminetetraacetic acid
RNA	Ribonucleic acid
g	rcf
PAM	Photospacer Adjacent Motifs
DSB	Double-Stranded Breaks
BAC	Bacterial Artificial Chromosome
NHEJ	Non-homologous end joining
HDR	Homologous Direct Repair

LB	Luria-Bertani
mM	Millimolar
µL	Microliter
ng	nanogram
V	Volt
ms	millisecond
PCR	Polymerase Chain Reaction
mg	milligram
CO2	Carbon Dioxide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
nm	nanometer
ANOVA	Analysis of Variant
TEM	Transfer Electron Microscopy
Ω	Omega
FLASH	Finding Low Abundance Sequence by Hybridization

CHAPTER 1

INTRODUCTION

1.1 Background

Antibacterial resistance has been a significant concern worldwide. Bacterial antibiotic resistance has developed at a fast rate over time. There are quite several bacteria that have been classified into the Multidrug MDR Resistance bacteria (MDR) group. These MDR organisms are a well-known threat to healthcare facilities, treatment, and scientists. causes even more threats in developing countries as the ions and antibiotic resistance co-existence of high infect presides.

MDR caused the inability and decreased activity of the third generation cephalosporins when they are used against these MDR organisms. Moges et al. (2021) collected samples from three hospitals and found that out of 121 (85.8%) out of 141 Gram-negative bacteria isolated are MDR organisms. Awoke et al. (2021) also reported that 130 (98.5%) out of 132 samples *of K. pneumoniae* that they isolated in 2018 and 2019 are MDR *K. pneumoniae*.

CDC, in earlier 2021, mentioned in their report that more than 2.8 million cases of antibiotic resistance occurred yearly in the US only and caused an estimated death of 35 000 lives. The estimated yearly stipend due to the infection of multidrug resistance bacteria is 4.6 billion per year in the United States, which includes the treatment that appears in the community and the hospital and, worst, both (Nelson et al., 2021). In their paper, this amount of money was spent for treatment against six groups of multidrug resistance bacteria, including the ESBL organisms. Other groups are Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *enterococcus* (VRE), Carbapenem-resistant *Enterobacterales* (CRE), Carbapenem-resistant *Acinetobacter* (CRA), and MDR *Pseudomonas*.

ESBL organisms confer resistance against all penicillins, cephalosporins, monobactam antibiotics, and combination antibiotic treatments such as sulbactam and culvanic acid (Parveen et al. 2011). These ESBL organisms exhibit resistance by tolerating the most commonly used beta-lactam antibiotics. They function by hydrolyzing the antibiotic compound's betalactam ring, thus inactivating them. These beta-lactamase producers are familiar with Gram-negative bacteria. They were first discovered in *K. pneumoniae* and transduced to *Escherichia coli*, as stated by Knothe et al. (1983). Bacteria are organisms that are constantly evolving, and they possess the ability to share their genetic material. Either within or crossed over species. There are a few methods of bacteria sharing their genetic material. These methods are transformation, transduction, and conjugation (Adekunle, 2012).

ESBL *K. pneumoniae* is a well-known pathogen that causes nosocomial infections, such as bacteremia and pneumonia (Fils et al., 2021). In addition, infections caused by carbapenem resistance *K. pneumoniae* are estimated to rise from 4% in 2005 to 29% in 2015, with most of the bacteria detected as ESBL *K. pneumoniae* (80%) (Fils et al., 2021). This bacterium has been reported to become an ESBL organism since the 1980s. Since then, ESBL *K. pneumoniae* have been grouped into several ESBL classes based on the ESBL gene that they carried. These classes are the ESBL *CTX* gene, ESBL *SHV* gene, ESBL *TEM* gene, or carbapenase resistance gene. *CTX, SHV*, and the *TEM* are genes grouping that makes a microbial organism become an ESBL organism.

This bacterium, also known as an opportunistic pathogen, caused severe infections in compromised patients in the hospital, especially those with underlying severe disease and immunocompromised patients (Parveen et al. 2011). ESBL *K. pneumoniae* often exhibits infection in a co-resistance manner toward antibiotics. This caused treatment of ESBL *K. pneumoniae* to become more challenging as fewer antibiotics can be used to treat them, and the option is limited (Lee et al. 2006). The current treatment employed the usage of either imipenem or meropenem against all ESBL organisms.

However, they may cause an allergic reaction in patients with carbapenem antibiotics sensitivity. Thus, it is crucial to find solutions for this ESBL organism without increasing its antibiotic resistance to a higher degree. It will also enhance the carbapenem *K. pneumoniae* resistance to a higher degree (Lee et al., 2006).

1.2 Problem Statement

There is now a new group of antibiotic resistance bacteria, the Extended Spectrum Beta Lactamase (ESBL) inhibitor organisms, a particular type of multidrug-resistant organisms. The scientific world is in a hurry to provide the best solution for these ESBL organisms. CDC (2021) reported that up to this moment, a few carbapenem class antibiotic antibiotics can still fight against ESBL organisms. However, with more and more treatment of the same antibiotic, the bacteria will become resistant as time goes by.

Respective bacteria will show their resistance in three ways; intrinsic, circumstantial, and acquired resistance when genetic codes are shared. Hence, when the bacterium has been treated with the same antibiotic for a period, it will become resistant to that specific type of antibiotic.

Thus, alternative treatments are in need to be developed. The alternative treatment will be a substitute for the antibiotic and helps control the ESBLs and the MDR organisms. At the same time will not enhance the bacteria resistance ability.

This research is proposed to employ CRISPR/Cas9 mechanisms against an Extended Spectrum Beta Lactamases producer, ESBL *K. pneumoniae* strain UPM ESBLKP1. It was isolated from a compromised, urinary tract-infected patient (Mohd Helmi et al., 2016) by disturbing their growth-related gene: the *uppP* gene. CRISPR/Cas9

mechanisms have been demonstrated to modify the genome of various organisms, such as animals, plants, fungi, and even bacteria.

CRISPR/Cas9 was identified as the adaptive immunity of bacteria against foreign invasion and has been used as a genetic engineering tool to modify target organisms to obtain better phenotypes. However, the study of manipulating the CRISPR/Cas9 system in bacteria against the bacteria itself is still lacking. Hence, this study will be carried out step by step by identifying a genomic region of the *uppP* gene similar to every ESBL *K. pneumoniae* and designing a corresponding guide RNA (gRNA) for targeting. Targets for modification are selected without affecting the *uppP* gene of probiotic bacteria.

uppP gene is selected because of the gene responsible for making up the bacteria's cell wall. This gene is underrated and always missed out in bacteria that are pathogenic to humans. However, this gene is used for many types of research that target bacteria modification and growth inhibition for bacteria that cause infection in plants, especially bacteria that cause infection in the root. These indicate that the *uppP* gene is also suitable for modification and treatment even though the bacteria host is pathogenic to humans instead of pathogenic to plants.

Research that has been performed by Kim et al. (2013) also showed a promising result of *uppP* modification of *Burkholderia* spp. bacteria against stinkbug insects. The loss of the *uppP* gene has caused the bacteria to become unable to localize and infect the stinkbug.

Thus, by modifying the *uppP* gene of the ESBL *K. pneumoniae* strain UPM ESBLKP1 the growth of the bacteria is disturbed due to loss of ability to express the protein required for the cell wall synthesis and growth, which subsequently destroy the bacteria without the usage of the antibiotic or drugs.

1.3 Hypothesis

The CRISPR technology that carries respective gRNAs, which target the uppP gene of the ESBL's *K. pneumoniae* UPM ESBL KP1, strain targeting the cell wall of the organisms will be delivered into *K. pneumoniae*, and the transformation will assist in the modification. In response to the modification of the *uppP* gene, it is expected that the formation of the bacteria cell wall will be disrupted and subsequently inhibited.

1.4 Objectives

1.4.1 General Objective

To utilize CRISPR/Cas9 technology that carries gRNAs designed to target the *uppP* gene in *K. pneumoniae* UPM ESBLKP1 strain for cell wall disruption and inhibition.

1.4.2 Specific Objectives

The specific objectives of this research are:

- To disrupt the peptidoglycan synthesis gene (*uppP* gene) of *K. pneumoniae* strain UPM ESBLKP1 using CRISPRCas/9 technology.
- To characterize the growth performance, cell wall integrity, and morphological changes of wild-type and mutant strains of the modified gene
- To evaluate the cytopathogenicity of the mutant strains in vitro.

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